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Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
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PITX2 - a marker to predict survival of patients diagnosed with breast cell proliferative disease

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Title

PITX2 - a marker to predict survival of patients diagnosed with breast cell proliferative disease

Abstract

The present invention relates to methods for predicting the survival of a human being diagnosed with a cell proliferative disorder of the breast tissues, characterized by a step of determining the expression level of PITX2 or the genetic or the epigenetic modifications of the genomic DNA associated with the gene PITX2.
The invention also relates to modified sequences, to oligonucleotides and/or PNA-oligomers which can be used within the described methods.

Field of the Invention

BREAST CANCER SURVIVAL

In European and American women, breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death. In women aged 40-55, breast cancer is the leading cause of death (Greenlee *et al.*, 2000). In 2002, there were 204,000 new cases of breast cancer in the US and a comparable number in Europe.

Breast cancer is defined as the uncontrolled proliferation of cells within breast tissues. Breasts are comprised of 15 to 20 lobes joined together by ducts. Cancer arises most commonly in the duct, but is also found in the lobes with the rarest type of cancer termed inflammatory breast cancer. It will be appreciated by those skilled in the art that there exists a continuing need to improve methods of early detection, classification and treatment of breast cancers. In contrast to the detection of some other common cancers such as cervical and dermal there are inherent difficulties in classifying and detecting breast cancers.

The first step of any treatment is the assessment of the patient's condition comparative to defined classifications of the disease. However the value of such a system is inherently dependent upon the quality of the classification. Breast cancers are staged according to their

size, location and occurrence of metastasis. Methods of treatment include the use of surgery, radiation therapy, chemotherapy and endocrine therapy, which are also used as adjuvant therapies to surgery. In general, more aggressive disease should be treated with more aggressive therapies.

Although the vast majority of early cancers are operable, i.e. the tumor can be completely removed by surgery, about one third of the patients with lymph-node negative diseases and about 50-60% of patients with node-positive disease will develop metastases during follow-up.

Based on this observation, systemic adjuvant treatment has been introduced for both node-positive and node-negative breast cancers. Systemic adjuvant therapy is administered after surgical removal of the tumor, and has been shown to reduce the risk of recurrence significantly. Several types of adjuvant treatment are available: endocrine treatment, also called hormone treatment (for hormone receptor positive tumors), different chemotherapy regimens, and antibody treatments based on novel agents like Herceptin (an antibody to an epidermal growth factor receptor).

The growth of the majority of breast cancers (appr. 70-80%) is dependent on the presence of estrogen. Therefore, one important target for adjuvant therapy is the removal of estrogen (e.g. by ovarian ablation) or the blocking of its synthesis or the blocking of its actions on the tumor cells either by blocking the receptor with competing substances (e.g. Tamoxifen) or by inhibiting the conversion of androgen into estrogen (e.g. aromatase inhibitors). Endocrine treatment is thought to be efficient only in tumors that express hormone receptors (the estrogen receptor (ER) and/or the progesterone receptor (PR)). Currently, the vast majority of women with hormone receptor positive breast cancer receive some form of endocrine treatment, independent of their nodal status. The most frequently used drug is Tamoxifen.

However, even in hormone receptor positive patients, not all patients benefit from endocrine treatment. Adjuvant endocrine therapy reduces mortality rates by 22% while response rates to endocrine treatment in the advanced setting are 50 to 60%.

Since Tamoxifen has relatively few side effects, treatment may be justified even for patients with low likelihood of benefit. However, these patients may require additional, more aggressive adjuvant treatment. Even in earliest and least aggressive tumors, such as node-negative, hormone receptor positive tumours, about 21% of patients relapse within 10 years after initial diagnosis if they receive Tamoxifen monotherapy only, as adjuvant treatment (Lancet. 1998 May 16;351(9114):1451-67. Tamoxifen for early breast cancer: an overview of

the randomised trials. Early Breast Cancer Trialists' Collaborative Group.). Similarly, some patients with hormone receptor negative disease may be treated sufficiently with surgery and potentially radiotherapy alone, whereas others may require additional chemotherapy.

Several cytotoxic regimens have shown to be effective in reducing the risk of relapse in breast cancer (Mansour *et al.*, 1998). According to current treatment guidelines, most node-positive patients receive adjuvant chemotherapy both in the US and Europe, since the risk of relapse is considerable. Nevertheless, not all patients do relapse, and there is a proportion of patients who would never have relapsed even without chemotherapy, but who nevertheless receive chemotherapy due to the currently used criteria. In hormone receptor positive patients, chemotherapy is usually given before endocrine treatment, whereas hormone receptor negative patients receive only chemotherapy.

The situation for node-negative patients is particularly complex. In the US, cytotoxic chemotherapy is recommended for node-negative patients, if the tumor is larger than 1 cm. In Europe, chemotherapy is considered for the node-negative cases if one or more risk factors such as tumor size larger than 2 cm, negative hormone receptor status, or tumor grading of three or age <35 is present. In general, there is a tendency to select premenopausal women for additional chemotherapy whereas for postmenopausal women, chemotherapy is often omitted. Compared to endocrine treatment, in particular Tamoxifen or aromatase inhibitors, chemotherapy is highly toxic, with short-term side effects such as nausea, vomiting, bone marrow depression, and long-term effects such as cardiotoxicity and an increased risk for secondary cancers.

It is currently not clear which breast cancer patients should be selected for more aggressive therapy and which would do well without additional aggressive treatment, and clinicians agree that there is a large need for proper selection of patients. The difficulty of selecting the right patients for chemotherapy, and the lack of suitable criteria is also reflected by a recent study which showed that chemotherapy is used much less frequently than recommended, based on data from the New Mexico Tumor registry (Du *et al.*, 2003). This study provides substantial evidence that there is a need for better selection of patients for chemotherapy or other, more aggressive forms of breast cancer therapy.

This invention is about a new biomarker, which can be used to solve the problem described above. Based on the observation that methylation of the gene PITX2 (also known as PTX2) in breast tumor tissue, obtained from the surgically removed tumor, or obtained from biopsy material prior to the removal, is correlated with the survival time of breast cancer patients treated with Tamoxifen monotherapy, we invented a tool allowing a better selection of patients for more aggressive therapy, for example a cytotoxic therapy (chemotherapy) (besides or instead of an endocrine treatment like treatment with Tamoxifen or aromatase inhibitors) for breast cancer patients.

It can be concluded that the expression levels of the protein or mRNA also correlate with said prognosis. Therefore, the analysis of either the expression levels of PITX2 protein, or PITX2 mRNA or the analysis of the patient's individual genetic or epigenetic modification of the gene PITX2 – summarized as the analysis of expression of the gene PITX2 - may serve as a method for predicting the survival of a patient with breast cancer. Especially the invention relates to methods for predicting the survival of a patient with breast cancer who is treated with at least one adjuvant endocrine treatment, wherein endocrine treatment is meant to comprise any treatment targeting the estrogen receptor pathway or estrogen synthesis pathway or estrogen conversion pathway i.e., which is involved in estrogen metabolism, production or secretion.

In this context survival is meant to describe the time from diagnosis or start of treatment to an endpoint, which may be the time of death (considering any reason for death or only death from breast cancer), or the time of recurrence of breast cancer, which may be local or distant, or the time of occurrence of any breast cancer associated disease. Therefore "predicting the survival" is meant to comprise predicting the disease free survival, as well as the overall survival or any other consideration of time between diagnosis and endpoint of treatment.

PITX2 (also known as PTX2) is known to belong to the PTX subfamily of PTX1, PTX2, and PTX3 genes which define a novel family of transcription factors, within the paired-like class of homeodomain factors. The gene PITX2 (NM_000325) encodes the paired-like homeodomain transcription factor 2, which is known to be expressed during development of anterior structures such as the eye, teeth, and anterior pituitary.

Toyota et *al.*, (2001) (Blood 97: p 2823-9.) found hypermethylation of the PITX2 gene in a large proportion of acute myeloid leukemias. Furthermore, in this study hypermethylation of PITX2 is positively correlated to methylation of the ER gene.

Although the expression of PITX2 is associated with cell differentiation and proliferation it has no heretofore recognized role in carcinogenesis of breast cancer or responsiveness to endocrine treatment.

EXPRESSION ANALYSIS

The expression of a gene, or rather the protein encoded by the gene, can be studied on four different levels: firstly, protein expression levels can be determined directly, secondly, mRNA transcription levels can be determined, thirdly, epigenetic modifications, such as gene's DNA methylation profile or the gene's histone profile; can be analyzed, as methylation is often correlated with inhibited protein expression, and fourth, the gene itself may be analyzed for genetic modifications such as mutations, deletions, polymorphisms etc. influencing the expression of the gene product.

The levels of observation that have been studied by the methodological developments of recent years in molecular biology, are the genes themselves, the transcription of these genes into RNA, and the translation into the resulting proteins. However how the activation and inhibition of specific genes, in specific cells and tissues, at specific time points in the course of development of an individual are controlled, is correlatable to the degree and character of the methylation of the genes or respectively the genome. In this respect, pathogenic conditions may manifest themselves in a changed methylation pattern of individual genes or of the genome.

The four terms that apply to the fields of overall genome-wide analysis of all these biological processes are called: Proteomics, Transcriptomics, Epigenomics (or Methylomics) and Genomics. Methods and techniques that can be used for studying expression or studying the modifications responsible for expression on all of these levels are well described in the literature and therefore known to a person skilled in the art. They are described in text books of molecular biology and in a large number of scientific journals.

How to analyze the protein expression of a single gene is prior art. It usually requires an antibody specific for the gene product of interest. Appropriate technologies would be ELISA or Immunohistochemistry.

The analysis of the level of mRNA also has been described sufficiently. These days the gold standard is the reverse transcriptase PCR.

To avoid duplication a more detailed description of the prior art relating to existing and well known technologies is given within the description of the invention, as it is part of the invention.

US patent application 2003/0198970 by Gareth Roberts lists some of the technologies and methods on how to determine a person's "genetic make up", i.e. the genetic modifications, such as deletions, polymorphisms, mutations etc. that may vary between individuals and describes the potential role of this genetic sequence information in the individual's variability in disease, response to therapy and prognosis. Epigenetic differences however are not mentioned. The gene PITX2 is listed within this application as one gene name out of a long and comprehensive list of about 2.500 other gene names, suggesting its expression could play a role in some kind of treatment response. However, this is simply an assumption based on speculation only, as no experiments are disclosed, which demonstrate any kind of relation between genetic modifications of PITX2 and an individual's variation in treatment response.

A less established area in this context is the field of epigenomics or epigenetics, i.e. the field concerned with analysis of DNA methylation patterns.

Methylation of DNA can play an important role in the control of gene expression in mammalian cells. DNA methyltransferases are involved in DNA methylation and catalyze the transfer of a methyl group from S-adenosylmethionine to cytosine residues to form 5-methylcytosine, a modified base that is found mostly at CpG sites in the genome. The presence of methylated CpG islands in the promoter region of genes can suppress their expression. This process may be due to the presence of 5-methylcytosine, which apparently interferes with the binding of transcription factors or other DNA-binding proteins to block transcription. In different types of tumors, aberrant or accidental methylation of CpG islands in the promoter region has been observed for many cancer-related genes, resulting in the silencing of their expression. Such genes include tumor suppressor genes, genes that suppress metastasis and angiogenesis, and genes that repair DNA (Momparler and Bovenzi (2000) J. Cell Physiol. 183:145-54).

In addition it has been described that DNA methylation may also play a role in the field of pharmacogenetics. A similar approach on how to apply information about genetic

modifications of the genome to the analysis of individual responses to treatment as was for example described by Gareth Roberts in US application 2003/0198970 was already subject of the application WO 02/037398, tailored to the application of information about *epigenetic* modifications of the genome, based on DNA methylation analysis, to guide treatment selection and to study individual's treatment responses.

An example for the applicability of this idea was given by Esteller et al. (Esteller et al. (2000) N Engl J Med. 2000 Nov 9;343(19):1350-4.), who demonstrated that methylation of the MGMT promoter in gliomas is a useful predictor of the responsiveness of the tumors to alkylating agents.

An example for the potential of analysis of epigenetic modifications, such as DNA methylation analysis, for the prediction of treatment response - related to breast cancer- was presented as a poster by Martens et al. at the San Antonio Breast Cancer Symposium, San Antonio, TX, December, 3-6, 2003. Breast cancer patients which have had their tumors removed by surgery and developed metastases at some point after the removal, were treated with Tamoxifen, an endocrine treatment drug. The primary tumor samples were analyzed for aberrant methylation patterns. The patients were then divided into two sub classes according to their objective tumor response: patients with progressive disease (which could be described as increasing metastasis size) and patients with complete or partial remission of the relapsed tumor (which could be described as decreasing metastasis size). It turned out, that those patients which had a tumor removed and experienced a remission (decrease in size) of the metastasis under endocrine treatment, had suffered from a tumor which showed a distinct pattern of DNA methylation at specific CpG sites, whereas patients which show progressive disease (did not experience a decrease but an increase in size of their metastases), under endocrine treatment, suffered from a tumor which did not show this distinct pattern of DNA methylation (but a different pattern) at these CpG sites. This is a clear indication, that the methylation pattern described in that study can serve as a **predictive** treatment response tool for an endocrine treatment, like tamoxifen. The results of this study, i.e. predictive biomarkers and assays therefore, are subject of patent application PCT/EP03/07827 [not yet published]: Method and nucleic acid for the analysis of breast cell proliferative disorders. Predictive markers as described above will also be called 'metastatic' markers in the context of this application.

Currently several predictive markers are under evaluation. As up to now most patients have received Tamoxifen as endocrine treatment most of the markers have been shown to be associated with response or resistance to tamoxifen. However, it is generally assumed that there is a large overlap between responders to one or the other endocrine treatment. In fact, ER and PR expression are used to select patients for any endocrine treatment. Among the markers which have been associated with TAM response is bcl-2. High bcl-2 levels showed promising correlation to TAM therapy response in patients with metastatic disease and prolonged survival and added valuable information to an ER negative patient subgroup (J Clin Oncology, 1997, 15 5: 1916-1922; Endocrine, 2000, 13(1):1-10). There is conflicting evidence regarding the independent predictive value of c-erbB2 (Her2/neu) overexpression in patients with advanced breast cancer that require further evaluation and verification (British J of Cancer, 1999, 79 (7/8):1220-1226; J Natl Cancer Inst, 1998, 90 (21): 1601-1608).

Other predictive markers include SRC-1 (steroid receptor coactivator-1), CGA gene over expression, cell kinetics and S phase fraction assays (Breast Cancer Res and Treat, 1998, 48:87-92; Oncogene, 2001, 20:6955-6959). Recently, uPA (Urokinase-type plasminogen activator) and PAI-1 (Plasminogen activator inhibitor type 1) together showed to be useful to define a subgroup of patients who have worse prognosis and who would benefit from adjuvant systemic therapy (J Clinical Oncology, 2002, 20 n° 4). However, all of these markers need further evaluations in prospective trials as none of them is yet a validated marker of response.

Also recently published was a study related to the **prognostic** power of methylation analysis in breast cancer patients. Müller et al. (Muller HM, Widschwendter A, Fiegl H, Ivarsson L, Goebel G, Perkmann E, Marth C, Widschwendter M. (2003) DNA methylation in serum of breast cancer patients: an independent prognostic marker. Cancer Res. 2003 Nov 15; 63(22): 7641-5.) reported about a set of genes, which can be used as biomarkers in patient pre-therapeutic sera for the prognosis of breast cancer. Specific aberrant methylation patterns of two genes found in DNA from pretreatment serum of cancer patients indicated whether their prognosis was good or bad. The DNA analyzed was not tumor DNA but serum DNA. Most likely the presence of a tumor-specific pattern indicates that tumor derived DNA is present, however, the absence of a specific methylation pattern, may be due to a tumor which does not show this methylation pattern, or a tumor which does not shed sufficient DNA into the blood

stream. Good or bad prognosis was defined as long or short "overall survival" after surgery, without adjuvant treatment. This result therefore relates to untreated patients, only.
These 'prognostic' markers are able to answer the question whether or not a breast cancer patient should get an aggressive adjuvant treatment like chemotherapy after removal of the tumor to avoid recurrence of cancer, i.e. occurrence of metastases.

However, none of these study results and none of these markers is able to answer the specific question raised above, whether or not a breast cancer patient should get adjuvant chemotherapy after removal of the tumor to avoid recurrence of cancer, i.e. occurrence of metastases in addition to endocrine treatment (with a drug like tamoxifen, or aromatase inhibitors).

A marker for a bad prognosis for cancer patients (without treatment), might not be applicable to a patient under adjuvant treatment with a drug like tamoxifen. Therefore the test would not be able to help deciding, whether chemotherapy, including all its side affects and inherent risks, is necessary or whether endocrine treatment is sufficient, because an endocrine treatment might change the prognosis from "bad" to "good".

The predictive 'metastatic' marker set described above, would be able to identify amongst all patient which relapse (develop metastases after surgery) those patients, which do not respond to endocrine treatment (by partial or complete remission of relapsed tumor). These markers however, cannot be applied to answer the question whether metastases will occur at all (after surgery of the primary tumor under endocrine treatment), and consequently whether it is advised to give adjuvant chemotherapy to avoid recurrence of cancer (i.e. relapse or occurrence of metastases).

In one aspect the present invention provides a marker, PITX2 (NM_000325), that can be used to answer that question and help guiding the decision whether or not an adjuvant chemotoxic therapy shall be subscribed in addition or instead of treatment with endocrines, like tamoxifen. A marker able to answer this question will also be called 'adjuvant' marker, in the context of this application.

In addition study results presented by Paik et al. at the San Antonio Breast Cancer Symposium, San Antonio, TX, December, 3-6, 2003 provide an answer to this question, by

analyzing the mRNA expression pattern of 16 genes with RT-PCR. They did not publish the identity of these 16 'adjuvant' markers.

For demonstration : The 'metastatic' test (use of a 'metastatic' marker) tells a patient that she will not respond to endocrine treatment when she develops metastases. But she does not know how high the likelihood is, that she will experience a relapse at all.
The 'prognostic' test (use of a 'prognostic' marker) tells a patient whether she will have a good or bad prognosis without any treatment. Even with a "bad prognosis" endocrine treatment might be enough.
The 'adjuvant test' (use of an 'adjuvant' marker) tells her whether she will or will not develop recurrence, without chemotherapy, even when treated with the standard -low side effected- endocrine treatment.

PITX2, however, which serves as an 'adjuvant marker' may also work as a 'prognostic marker', especially in hormone receptor negative women, which would not get any endocrine treatment at all.

5-methylcytosine is the most frequent covalent base modification in the DNA of eukaryotic cells. It plays a role, for example, in the regulation of the transcription, in genetic imprinting, and in tumorigenesis. Therefore, the identification of 5-methylcytosine as a component of genetic information is of considerable interest. However, 5-methylcytosine positions cannot be identified by sequencing since 5-methylcytosine has the same base pairing behaviour as cytosine. Moreover, the epigenetic information carried by 5-methylcytosine is completely lost during PCR amplification.

A relatively new and currently the most frequently used method for analysing DNA for 5-methylcytosine is based upon the specific reaction of bisulfite with cytosine which, upon subsequent alkaline hydrolysis, is converted to uracil which corresponds to thymidine in its base pairing behaviour. However, 5-methylcytosine remains unmodified under these conditions. Consequently, the original DNA is converted in such a manner that methylcytosine, which originally could not be distinguished from cytosine by its hybridisation behaviour, can now be detected as the only remaining cytosine using "normal" molecular biological techniques, for example, by amplification and hybridisation or sequencing. All of

these techniques are based on base pairing which can now be fully exploited. In terms of sensitivity, the prior art is defined by a method which encloses the DNA to be analysed in an agarose matrix, thus preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and which replaces all precipitation and purification steps with fast dialysis (Olek A, Oswald J, Walter J. A modified and improved method for bisulphite based cytosine methylation analysis. Nucleic Acids Res. 1996 Dec 15;24(24):5064-6). Using this method, it is possible to analyse individual cells, which illustrates the potential of the method. However, currently only individual regions of a length of up to approximately 3000 base pairs are analysed, a global analysis of cells for thousands of possible methylation events is not possible. However, this method cannot reliably analyse very small fragments from small sample quantities either. These are lost through the matrix in spite of the diffusion protection.

An overview of the further known methods of detecting 5-methylcytosine may be gathered from the following review article: Rein, T., DePamphilis, M. L., Zorbas, H., Nucleic Acids Res. 1998, 26, 2255.

To date, barring few exceptions (e.g., Zeschnigk M, Lich C, Buiting K, Doerfler W, Horsthemke B. A single-tube PCR test for the diagnosis of Angelman and Prader-Willi syndrome based on allelic methylation differences at the SNRPN locus. Eur J Hum Genet. 1997 Mar-Apr;5(2):94-8) the bisulfite technique is only used in research. Always, however, short, specific fragments of a known gene are amplified subsequent to a bisulfite treatment and either completely sequenced (Olek A, Walter J. The pre-implantation ontogeny of the H19 methylation imprint. Nat Genet. 1997 Nov;17(3):275-6) or individual cytosine positions are detected by a primer extension reaction (Gonzalgo ML, Jones PA. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). Nucleic Acids Res. 1997 Jun 15;25(12):2529-31, WO 95/00669) or by enzymatic digestion (Xiong Z, Laird PW. COBRA: a sensitive and quantitative DNA methylation assay. Nucleic Acids Res. 1997 Jun 15;25(12):2532-4). In addition, detection by hybridisation has also been described (Olek et al., WO 99/28498).

Further publications dealing with the use of the bisulfite technique for methylation detection in individual genes are: Grigg G, Clark S. Sequencing 5-methylcytosine residues in genomic DNA. Bioessays. 1994 Jun;16(6):431-6, 431; Zeschnigk M, Schmitz B, Dittrich B, Buiting K,

Horsthemke B, Doerfler W. Imprinted segments in the human genome: different DNA methylation patterns in the Prader-Willi/Angelman syndrome region as determined by the genomic sequencing method. Hum Mol Genet. 1997 Mar;6(3):387-95; Feil R, Charlton J, Bird AP, Walter J, Reik W. Methylation analysis on individual chromosomes: improved protocol for bisulphite genomic sequencing. Nucleic Acids Res. 1994 Feb 25;22(4):695-6; Martin V, Ribieras S, Song-Wang X, Rio MC, Dante R. Genomic sequencing indicates a correlation between DNA hypomethylation in the 5' region of the pS2 gene and its expression in human breast cancer cell lines. Gene. 1995 May 19;157(1-2):261-4; WO 97/46705 and WO 95/15373.

An overview of the Prior Art in oligomer array manufacturing can be gathered from a special edition of Nature Genetics (Nature Genetics Supplement, Volume 21, January 1999), published in January 1999, and from the literature cited therein.

Fluorescently labelled probes are often used for the scanning of immobilised DNA arrays. The simple attachment of Cy3 and Cy5 dyes to the 5'-OH of the specific probe are particularly suitable for fluorescence labels. The detection of the fluorescence of the hybridised probes may be carried out, for example via a confocal microscope. Cy3 and Cy5 dyes, besides many others, are commercially available.

Matrix Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas M, Hillenkamp F. Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. Anal Chem. 1988 Oct 15;60(20):2299-301). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapour phase in an unfragmented manner. The analyte is ionised by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones.

MALDI-TOF spectrometry is excellently suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut I G, Beck S. DNA and Matrix Assisted Laser Desorption Ionization Mass Spectrometry. Current Innovations and Future Trends. 1995, 1; 147-57). The sensitivity to nucleic acids is approximately 100 times worse

than to peptides and decreases disproportionally with increasing fragment size. For nucleic acids having a multiply negatively charged backbone, the ionisation process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallisation. There are now several responsive matrixes for DNA, however, the difference in sensitivity has not been reduced. The difference in sensitivity can be reduced by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. Phosphorothioate nucleic acids in which the usual phosphates of the backbone are substituted with thiophosphates can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut IG, Beck S. A procedure for selective DNA alkylation and detection by mass spectrometry. Nucleic Acids Res. 1995 Apr 25;23(8):1367-73). The coupling of a charge tag to this modified DNA results in an increase in sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities which make the detection of unmodified substrates considerably more difficult.

Genomic DNA is obtained from DNA of cell, tissue or other test samples using standard methods. This standard methodology is found in references such as Sambrook, Fritsch and Maniatis eds., Molecular Cloning: A Laboratory Manual, 1989.

DESCRIPTION

Characterisation of a breast cancer in terms of its predicted aggressiveness enables the physician to make an informed decision as to a therapeutic regimen with appropriate risk and benefit trade offs to the patient. Aggressiveness is taken to mean one or more of decreased patient survival or disease- or relapse-free survival, increased tumor-related complications and faster progression of tumor or metastases. According to the aggressiveness of the disease an appropriate treatment or treatments may be selected from the group consisting of chemotherapy, radiotherapy, surgery, biological therapy, immunotherapy, antibody treatments, treatments involving molecularly targeted drugs, estrogen receptor modulator treatments, estrogen receptor down-regulator treatments, aromatase inhibitors treatments, ovarian ablation, treatments providing LHRH analogues or other centrally acting drugs influencing estrogen production. Wherein a cancer is characterised as 'aggressive' it is particularly preferred that a treatment such as, but not limited to, chemotherapy is provided in addition to or instead of an endocrine targeting therapy.

Using the methods and nucleic acids described herein, statistically significant models of patient disease free or overall survival and/or disease progression can be developed and utilised to assist patients and clinicians in determining suitable treatment options to be included in the therapeutic regimen.

In one aspect the described method is to be used to assess the utility of therapeutic regimens comprising one or more treatments which is either an aggressive therapy such as chemotherapy or a treatment which targets the estrogen receptor pathway or is involved in estrogen metabolism, production or secretion as a therapy for patients suffering from a cell proliferative disorder of the breast tissues. In particular this aspect of the method enables the physician to determine which treatments may be used in addition to or instead of said endocrine treatment.

In a further aspect the described method enables the characterisation of the cell proliferative disorder in terms of agressiveness, thereby enabling the physician to recommend suitable treatments. Thus, the present invention will be seen to reduce the problems associated with present breast cell proliferative disorder treatment response prediction methods.

Using the methods and nucleic acids as described herein, patient survival can be evaluated before or during treatment for a cell proliferative disorder of the breast tissues, in order to provide critical information to the patient and clinician as to the likely progression of the disease. It will be appreciated, therefore, that the methods and nucleic acids exemplified herein can serve to improve a patient's quality of life and odds of treatment success by allowing both patient and clinician a more accurate assessment of the patient's treatment options.

The method according to the definition may be used for the improved treatment of all breast cell proliferative disorder patients, both pre and post menopausal and independent of their node or estrogen receptor status. However, it is particularly preferred that said patients are node-negative and estrogen receptor positive.

The present invention makes available a method for the improved treatment and monitoring of breast cell proliferative disorders, by enabling the accurate prediction of a patient's survival without systemic therapy or with endocrine therapy comprising one or more treatments which target the estrogen receptor pathway or are involved in estrogen metabolism, production, or secretion.

In a particularly preferred embodiment, the method according to the invention enables the differentiation between patients who have a high risk of relapse under said endocrine therapy and those who have a low risk of relapse under said therapy.
The method particularly preferably enables the determination of a methylation pattern characteristic for a predicted survival time, in addition to the characterisation of tumors in terms of aggressiveness.

The method according to the invention may be used for the analysis of a wide variety of cell proliferative disorders of the breast tissues including, but not limited to, ductal carcinoma *in situ*, invasive ductal carcinoma, invasive lobular carcinoma, lobular carcinoma *in situ*, comedocarcinoma, inflammatory carcinoma, mucinous carcinoma, scirrhous carcinoma, colloid carcinoma, tubular carcinoma, medullary carcinoma, metaplastic carcinoma, and papillary carcinoma and papillary carcinoma *in situ*, undifferentiated or anaplastic carcinoma and Paget's disease of the breast.

The method according to the invention is particularly suited to the prediction of survival of breast cancer in the following treatment setting. In one embodiment, the method is applied to patients who receive endocrine pathway targeting treatment as secondary treatment to an initial non chemotherapeutical therapy, e.g. surgery (hereinafter referred to as the adjuvant setting) as illustrated in Figure 1. Such a treatment is often prescribed to patients suffering from Stage 1 to 3 breast carcinomas. In this embodiment patients survival times are predicted according to their gene expression or genetic or epigenetic modifications. By detecting patients with worse disease free survival times the physician may choose to recommend the patient for further treatment, instead of or in addition to the endocrine targeting therapy(s), in particular but not limited to, chemotherapy.

This invention is specifically about a new biomarker, PITX2, for patients diagnosed with breast cell proliferative disease, allowing the prediction of outcome without treatment, or with different therapies, like a cytotoxic therapy (chemotherapy) in addition or instead of (for example in hormone receptor negative patients) an endocrine treatment, like treatment with Tamoxifen or aromatase inhibitors, wherein the prediction is based on the patient's survival or clinical or pathological tumor response, or response measured with other surrogate parameters.

This invention therefore related to new methods or tools, for patients diagnosed with breast cell proliferative disease, allowing the evaluation of adjuvant therapy based on a prediction of outcome.

More specifically this invention provides new methods or tools, for patients diagnosed with breast cell proliferative disease, allowing the evaluation of adjuvant therapy, i.e. therapy after surgical removal of the tumor, like a cytotoxic therapy (chemotherapy) in addition to or instead of (for example in hormone receptor negative patients) an endocrine treatment, like treatment with Tamoxifen or aromatase inhibitors, wherein the evaluation is based on the prediction of the patient's survival.

One aspect of the invention is the provision of tools for predicting the survival of a patient diagnosed with a breast cell proliferative disease, such as breast cancer. These tools comprise methods for the analysis of either the expression levels of PITX2 protein, or PITX2 mRNA or the analysis of the patient's individual genetic or epigenetic modification of the gene PITX2 – summarized as the analysis of expression of the gene PITX2. Preferably the invention relates to methods for predicting the survival of a patient diagnosed with breast cancer. Preferably said patient is treated with at least one adjuvant endocrine treatment, wherein endocrine treatment is meant to comprise any treatment targeting the estrogen receptor pathway or estrogen synthesis pathway or estrogen conversion pathway i.e., which is involved in estrogen metabolism, production or secretion. Preferably the patient was treated with said adjuvant endocrine treatment after surgical removal of the tumor. Also preferably the survival is the disease free survival.

Especially preferred are methods applied for the prediction of the disease free survival of a patient diagnosed with breast cancer under adjuvant endocrine treatment after surgical tumor removal. Even more preferred are those methods, which analyze the DNA methylation profile of the genomic region associated with the gene PITX2. Especially preferred is the analysis of the DNA methylation profile of the genomic sequence given in SEQ ID 1.

This methodology presents further improvements over the state of the art in that the method may be applied to any subject, independent of the estrogen and/or progesterone receptor status. Therefore in a preferred embodiment, the subject is not required to have been tested for estrogen or progesterone receptor status.

The object of the invention is achieved by means of the analysis of the methylation pattern of PITX2 and/or its regulatory region. In a particularly preferred embodiment the sequence of said gene comprises SEQ ID 1 and the sequence complementary thereto.

In one embodiment the object of the invention is the prediction of survival under a treatment which targets the estrogen receptor pathway or is involved in estrogen metabolism, production or secretion. This is achieved by analysis of the expression pattern of PITX2 and wherein it is further preferred that the sequence of said gene comprises SEQ ID NO: 1 .

In one aspect the invention discloses novel methods utilizing the gene PITX2 for the prediction of survival of a patient diagnosed with a breast cell proliferative disease. In a preferred embodiment said patient diagnosed with a breast cell proliferative disease is treated with adjuvant endocrine monotherapy.

The invention discloses the use of the gene PITX2, as well as its promoter and regulatory elements as a prognostic marker for survival of breast cancer patients. It is preferred that these patients are treated with adjuvant endocrine monotherapy. More specifically, the disclosed matter shows the applicability of said gene to answer the question and help guiding the decision whether or not an adjuvant chemotoxic therapy shall be subscribed, preferably in addition of endocrine treatment, like the treatment with tamoxifen or aromatase inhibitors.

In one aspect of the invention, the disclosed matter provides novel nucleic acid sequences useful for the analysis of methylation within said gene, other aspects provide novel uses of the gene and the gene product as well as methods, assays and kits directed to prognosing the survival of a patient diagnosed with breast cell proliferative disease. Preferably a patient which is treated with adjuvant endocrine monotherapy.

In one embodiment the method discloses the use of the gene PITX2 as a marker for the prognosis of the survival of a patient suffering from a breast cell proliferative disease. Preferably said patient is treated with adjuvant endocrine monotherapy. Said use of the gene may be enabled by means of any analysis of the expression of the gene, by means of mRNA expression analysis or protein expression analysis or by analysis of its genetic modifications leading to an altered expression.

However, in the most preferred embodiment of the invention, prediction of the survival of a patient diagnosed with breast cell proliferative disease, preferably treated with adjuvant endocrine monotherapy, is enabled by means of analysis of the methylation status of CpG sites within the gene PITX2 and its promoter or regulatory elements.

To detect the presence of mRNA encoding PITX2 in a detection system for breast cancer relapse, a sample is obtained from a patient. The sample can be a tumor tissue sample from the surgically removed tumor, a biopsy sample or a sample of blood, plasma, serum or the like. The sample may be treated to extract the nucleic acids contained therein. The resulting nucleic acid from the sample is subjected to gel electrophoresis or other separation techniques. Detection involves contacting the nucleic acids and in particular the mRNA of the sample with a DNA sequence serving as a probe to form hybrid duplexes. The stringency of hybridisation is determined by a number of factors during hybridisation and during the washing procedure, including temperature, ionic strength, length of time and concentration of formamide. These factors are outlined in, for example, Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd ed., 1989). Detection of the resulting duplex is usually accomplished by the use of labelled probes. Alternatively, the probe may be unlabeled, but may be detectable by specific binding with a ligand which is labelled, either directly or indirectly. Suitable labels and methods for labelling probes and ligands are known in the art, and include, for example, radioactive labels which may be incorporated by known methods (e.g., nick translation or kinasing), biotin, fluorescent groups, chemiluminescent groups (e.g., dioxetanes, particularly triggered dioxetanes), enzymes, antibodies, and the like.

In order to increase the sensitivity of the detection in a sample of mRNA encoding PITX2, the technique of reverse transcription/polymerisation chain reaction can be used to amplify cDNA transcribed from mRNA encoding PITX2. The method of reverse transcription /PCR is well known in the art (for example, see Watson and Fleming, supra).

The reverse transcription /PCR method can be performed as follows. Total cellular RNA is isolated by, for example, the standard guanidium isothiocyanate method and the total RNA is reverse transcribed. The reverse transcription method involves synthesis of DNA on a template of RNA using a reverse transcriptase enzyme and a 3' end primer. Typically, the primer contains an oligo(dT) sequence. The cDNA thus produced is then amplified using the PCR method and PITX2 specific primers. (Belyavsky et al, Nucl Acid Res 17:2919-2932,

1989; Krug and Berger, Methods in Enzymology, Academic Press,N.Y., Vol.152, pp. 316-325, 1987 which are incorporated by reference)

The present invention may also be described in certain embodiments as a kit for use in prognosing the survival of a breast cancer patient before or after surgical tumor removal with or without adjuvant endocrine monotherapy state through testing of a biological sample. A representative kit may comprise one or more nucleic acid segments as described above that selectively hybridise to PITX2 mRNA and a container for each of the one or more nucleic acid segments. In certain embodiments the nucleic acid segments may be combined in a single tube. In further embodiments, the nucleic acid segments may also include a pair of primers for amplifying the target mRNA. Such kits may also include any buffers, solutions, solvents, enzymes, nucleotides, or other components for hybridisation, amplification or detection reactions. Preferred kit components include reagents for reverse transcription-PCR, in situ hybridisation, Northern analysis and/or RPA.

The present invention further provides for methods to detect the presence of the polypeptide, PITX2, in a sample obtained from a patient. Any method known in the art for detecting proteins can be used. Such methods include, but are not limited to immunodiffusion, immunoelectrophoresis, immunochemical methods, binder-ligand assays, immunohistochemical techniques, agglutination and complement assays. (for example see Basic and Clinical Immunology, Sites and Terr, eds., Appleton & Lange, Norwalk, Conn. pp 217-262, 1991 which is incorporated by reference). Preferred are binder-ligand immunoassay methods including reacting antibodies with an epitope or epitopes of PITX2 and competitively displacing a labelled PITX2 protein or derivative thereof.

Certain embodiments of the present invention comprise the use of antibodies specific to the polypeptide encoded by the PITX2 gene. Such antibodies may be useful for prognosing the survival of a breast cancer patient preferably under adjuvant endocrine monotherapy by comparing a patient's levels of PITX2 marker expression to expression of the same marker in normal individuals. In certain embodiments production of monoclonal or polyclonal antibodies can be induced by the use of the PITX2 polypeptide as antigene. Such antibodies may in turn be used to detect expressed proteins as markers for prognosis of relapse of a breast cancer patient under adjuvant endocrine monotherapy. The levels of such proteins present in the peripheral blood of a patient may be quantified by conventional methods.

Antibody-protein binding may be detected and quantified by a variety of means known in the art, such as labelling with fluorescent or radioactive ligands. The invention further comprises kits for performing the above-mentioned procedures, wherein such kits contain antibodies specific for the PITX2 polypeptides.

Numerous competitive and non-competitive protein binding immunoassays are well known in the art. Antibodies employed in such assays may be unlabeled, for example as used in agglutination tests, or labelled for use a wide variety of assay methods. Labels that can be used include radionuclides, enzymes, fluorescers, chemiluminescers, enzyme substrates or co-factors, enzyme inhibitors, particles, dyes and the like for use in radioimmunoassay (RIA), enzyme immunoassays, e.g., enzyme-linked immunosorbent assay (ELISA), fluorescent immunoassays and the like. Polyclonal or monoclonal antibodies to PITX2 or an epitope thereof can be made for use in immunoassays by any of a number of methods known in the art. One approach for preparing antibodies to a protein is the selection and preparation of an amino acid sequence of all or part of the protein, chemically synthesising the sequence and injecting it into an appropriate animal, usually a rabbit or a mouse (Milstein and Kohler Nature 256:495-497, 1975; Gulfre and Milstein, Methods in Enzymology: Immunochemical Techniques 73:1-46, Langone and Banatis eds., Academic Press, 1981 which are incorporated by reference). Methods for preparation of PITX2 or an epitope thereof include, but are not limited to chemical synthesis, recombinant DNA techniques or isolation from biological samples.

The invention provides significant improvements over the state of the art in that there are currently no markers known to the public which can be used to detect the likelihood of relapse or of survival of a breast cancer patient under adjuvant endocrine monotherapy, neither from tissue samples nor from body fluid samples.
Also, no methylation marker is known which can be used to detect the likelihood of relapse or of survival of a breast cancer patient. Especially, no methylation marker is known which can be used to detect the likelihood of relapse or of survival of a breast cancer patient under adjuvant endocrine monotherapy, neither from tissue samples nor from body fluid samples.

The objective of the invention can also be achieved by analysis of the methylation state of the CpG dinucleotides within the genomic sequence according to SEQ ID NO: 1 and sequences complementary thereto. SEQ ID NO: 1 discloses the gene PITX2 and its promoter and

regulatory elements, wherein said fragment comprises CpG dinucleotides exhibiting a disease specific methylation pattern. The methylation pattern of the gene PITX2 and its promoter and regulatory elements have heretofore not been analysed with regard to prognosis of survival of a patient diagnosed with a breast cell proliferative disorder . Due to the degeneracy of the genetic code, the sequence as identified in SEQ ID NO: 1 should be interpreted so as to include all substantially similar and equivalent sequences upstream of the promoter region of a gene which encodes a polypeptide with the biological activity of that encoded by PITX2.

In a preferred embodiment of the method, the objective of the invention is achieved by analysis of a nucleic acid comprising a sequence of at least 18 bases in length according to one of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

The sequences of SEQ ID NOS: 2 to 5 provide modified versions of the nucleic acid according to SEQ ID NO: 1, wherein the conversion of said sequence results in the synthesis of a nucleic acid having a sequence that is unique and distinct from SEQ ID NO: 1 as follows. (*see also* the following TABLE 1): SEQ ID NO: 1, sense DNA strand of PITX2 gene and its promoter and regulatory elements; SEQ ID NO: 2, converted SEQ ID NO: 1, wherein "C" converted to "T," but "CpG" remains "CpG." (*i.e.*, corresponds to case where, for SEQ ID NO: 1, all "C" residues of CpG dinucleotide sequences are methylated and are thus not converted); SEQ ID NO: 3, complement of SEQ ID NO: 1, wherein "C" converted to "T," but "CpG" remains "CpG" (*i.e.*, corresponds to case where, for the complement (*anti*sense strand) of SEQ ID NO: 1, all "C" residues of CpG dinucleotide sequences are methylated and are thus not converted); SEQ ID NO: 4, converted SEQ ID NO: 1, wherein "C" converted to "T" for all "C" residues, including those of "CpG" dinucleotide sequences (*i.e.*, corresponds to case where, for SEQ ID NO: 1, all "C" residues of CG dinucleotide sequences are unmethylated); SEQ ID NO: 5, complement of SEQ ID NO: 1, wherein "C" converted to"T" for all "C" residues, including those of "CpG" dinucleotide sequences (*i.e.*, corresponds to case where, for the complement (*anti*sense strand) of SEQ ID NO: 1, all "C" residues of CpG dinucleotide sequences are unmethylated).

TABLE 1. Description of SEQ ID NOS: 1 to 5

SEQ ID NO	Relationship to SEQ ID NO:1	Nature of cytosine base conversion
SEQ ID NO:1	Sense strand (PITX2 gene including promoter and regulatory elements)	None; untreated sequence
SEQ ID NO:2	Converted sense strand	"C"to"T," but "CpG" remains "CpG" (all "C" residues of CpGs are methylated)
SEQ ID NO:3	Converted antisense strand	"C"to"T," but "CpG" remains "CpG" (all "C" residues of CpGs are methylated)
SEQ ID NO:4	Converted sense strand	"C"to"T" for all "C" residues (all "C" residues of CpGs are unmethylated)
SEQ ID NO:5	Converted antisense strand	"C"to"T" for all "C" residues (all "C" residues of CpGs are unmethylated)

Significantly, heretofore, the nucleic acid sequences and molecules according to SEQ ID NO: 1 to SEQ ID NO: 5 were not implicated in or connected with the ascertainment of the prognosis of breast cancer relapse or survival.

The described invention further discloses oligonucleotides or oligomers for detecting the cytosine methylation state within pretreated DNA, according to SEQ ID NO: 2 to SEQ ID NO: 5. Said oligonucleotides or oligomers comprising a nucleic acid sequence having a length of at least nine (9) nucleotides which hybridise, under moderately stringent or stringent conditions (as defined herein above), to a pretreated nucleic acid sequence according to SEQ ID NO: 2 to SEQ ID NO: 5 and/or sequences complementary thereto.

Thus, the present invention includes nucleic acid molecules (*e.g.*, oligonucleotides and peptide nucleic acid (PNA) molecules (PNA-oligomers)) that hybridise under moderately stringent and/or stringent hybridisation conditions to all or a portion of the sequences of SEQ ID NOS: 2 to 5, or to the complements thereof. The hybridising portion of the hybridising nucleic acids is typically at least 9, 15, 20, 25, 30 or 35 nucleotides in length. However, longer molecules have inventive utility, and are thus within the scope of the present invention.

Preferably, the hybridising portion of the inventive hybridising nucleic acids is at least 95%, or at least 98%, or 100% identical to the sequence, or to a portion thereof of SEQ ID NOS: 2 to 5, or to the complements thereof.

Hybridising nucleic acids of the type described herein can be used, for example, as a primer (*e.g.*, a PCR primer), or a diagnostic and/or prognostic probe or primer. Preferably, hybridisation of the oligonucleotide probe to a nucleic acid sample is performed under stringent conditions and the probe is 100% identical to the target sequence. Nucleic acid duplex or hybrid stability is expressed as the melting temperature or Tm, which is the temperature at which a probe dissociates from a target DNA. This melting temperature is used to define the required stringency conditions.

For target sequences that are related and substantially identical to the corresponding sequence of SEQ ID NO: 1 (such as PITX2 allelic variants and SNPs), rather than identical, it is useful to first establish the lowest temperature at which only homologous hybridisation occurs with a particular concentration of salt (*e.g.*, SSC or SSPE). Then, assuming that 1% mismatching results in a 1°C decrease in the Tm, the temperature of the final wash in the hybridisation reaction is reduced accordingly (for example, if sequences having > 95% identity with the probe are sought, the final wash temperature is decreased by 5°C). In practice, the change in Tm can be between 0.5°C and 1.5°C per 1% mismatch.

Examples of inventive oligonucleotides of length X (in nucleotides), as indicated by polynucleotide positions with reference to, *e.g.*, SEQ ID NO: 1, include those corresponding to sets of consecutively overlapping oligonucleotides of length X, where the oligonucleotides within each consecutively overlapping set (corresponding to a given X value) are defined as the finite set of Z oligonucleotides from nucleotide positions:

n to (n + (X-1));

where n=1, 2, 3,...(Y-(X-1));

where Y equals the length (nucleotides or base pairs) of SEQ ID NO: 1 ;

where X equals the common length (in nucleotides) of each oligonucleotide in the set (*e.g.*, X=20 for a set of consecutively overlapping 20-mers); and

where the number (Z) of consecutively overlapping oligomers of length X for a given SEQ ID NO of length Y is equal to Y-(X-1). For example Z=2,785-19=2,766 for either sense or antisense sets of SEQ ID NO: 1, where X=20.

Preferably, the set is limited to those oligomers that comprise at least one CpG, Cpa or tpG dinucleotide, wherein 'Cpa' is indicating that said Cpa hybridises to a position (tpG) which was a CpG prior to bisulfite conversion and is a TpG now; and wherein 'tpG' is indicating that said tpG hybridises to a position (Cpa) which is the complementary to a position (tpG) which was a CpG prior to bisulfite conversion and is a TpG now.

The present invention encompasses, for *each* of SEQ ID NOS: 2 to 5 (sense and antisense), multiple consecutively overlapping sets of oligonucleotides or modified oligonucleotides of length X, where, *e.g.*, X= 9, 10, 17, 20, 22, 23, 25, 27, 30 or 35 nucleotides.

The oligonucleotides or oligomers according to the present invention constitute effective tools useful to ascertain genetic and epigenetic parameters of the genomic sequence corresponding to SEQ ID NO: 1. Preferred sets of such oligonucleotides or modified oligonucleotides of length X are those consecutively overlapping sets of oligomers corresponding to SEQ ID NOS:1-5 (and to the complements thereof). Preferably, said oligomers comprise at least one CpG, tpG or Cpa dinucleotide.

Particularly preferred oligonucleotides or oligomers according to the present invention are those in which the cytosine of the CpG dinucleotide (or of the corresponding converted TpG or CpA dinculeotide) sequences is within the middle third of the oligonucleotide; that is, where the oligonucleotide is, for example, 13 bases in length, the CpG, TpG or CpA dinucleotide is positioned within the fifth to ninth nucleotide from the 5'-end.

The oligonucleotides of the invention can also be modified by chemically linking the oligonucleotide to one or more moieties or conjugates to enhance the activity, stability or detection of the oligonucleotide. Such moieties or conjugates include chromophores, fluorophors, lipids such as cholesterol, cholic acid, thioether, aliphatic chains, phospholipids, polyamines, polyethylene glycol (PEG), palmityl moieties, and others as disclosed in, for example, United States Patent Numbers 5,514,758, 5,565,552, 5,567,810, 5,574,142, 5,585,481, 5,587,371, 5,597,696 and 5,958,773. The probes may also exist in the form of a PNA (peptide nucleic acid) which has particularly preferred pairing properties. Thus, the oligonucleotide may include other appended groups such as peptides, and may include hybridisation-triggered cleavage agents (Krol et al., *BioTechniques* 6:958-976, 1988) or

intercalating agents (Zon, *Pharm. Res.* 5:539-549, 1988). To this end, the oligonucleotide may be conjugated to another molecule, *e.g.*, a chromophore, fluorophor, peptide, hybridisation-triggered cross-linking agent, transport agent, hybridisation-triggered cleavage agent, etc.

The oligonucleotide may also comprise at least one art-recognized modified sugar and/or base moiety, or may comprise a modified backbone or non-natural internucleoside linkage.

The oligomers according to the present invention are normally used in so called "sets" which contain at least one oligomer for analysis of each of the CpG dinucleotides of a genomic sequence comprising SEQ ID NO: 1 and sequences complementary thereto or to their corresponding CG, tG or Ca dinucleotide within the pretreated nucleic acids according to SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto, wherein a 't' indicates a nucleotide which converted from a cytosine into a thymine and wherein 'a' indicates the complementary nucleotide to such a converted thymine. Preferred is a set which contains at least one oligomer for each of the CpG dinucleotides within the gene PITX2 and it's promoter and regulatory elements in both the pretreated and genomic versions of said gene, SEQ ID NO: 2 to 5 and SEQ ID NO: 1, respectively. However, it is anticipated that for economic or other factors it may be preferable to analyse a limited selection of the CpG dinucleotides within said sequences and the contents of the set of oligonucleotides should be altered accordingly. Therefore, the present invention moreover relates to a set of at least 3 n (oligonucleotides and/or PNA-oligomers) used for detecting the cytosine methylation state in pretreated genomic DNA (SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto) and genomic DNA (SEQ ID NO: 1 and sequences complementary thereto). These probes enable diagnosis and/or therapy of genetic and epigenetic parameters of cell proliferative disorders. The set of oligomers may also be used for detecting single nucleotide polymorphisms (SNPs) in pretreated genomic DNA (SEQ ID NO: 2 to SEQ ID NO: 5, and sequences complementary thereto) and genomic DNA (SEQ ID NO: 1, and sequences complementary thereto) .

Moreover, the present invention makes available a set of at least two oligonucleotides which can be used as so-called "primer oligonucleotides" for amplifying DNA sequences of one of SEQ ID NO: 1 to SEQ ID NO: 5 and sequences complementary thereto, or segments thereof.

In the case of the sets of oligonucleotides according to the present invention, it is preferred that at least one and more preferably all members of the set of oligonucleotides is bound to a solid phase.

According to the present invention, it is preferred that an arrangement of different oligonucleotides and/or PNA-oligomers (a so-called "array") made available by the present invention is present in a manner that it is likewise bound to a solid phase. This array of different oligonucleotide- and/or PNA-oligomer sequences can be characterised in that it is arranged on the solid phase in the form of a rectangular or hexagonal lattice. The solid phase surface is preferably composed of silicon, glass, polystyrene, aluminium, steel, iron, copper, nickel, silver, or gold. However, nitrocellulose as well as plastics such as nylon which can exist in the form of pellets or also as resin matrices may also be used.

Therefore, a further subject matter of the present invention is a method for manufacturing an array fixed to a carrier material for analysis in connection with cell proliferative disorders, in which method at least one oligomer according to the present invention is coupled to a solid phase. Methods for manufacturing such arrays are known, for example, from US Patent 5,744,305 by means of solid-phase chemistry and photolabile protecting groups.

A further subject matter of the present invention relates to a DNA chip for the analysis of cell proliferative disorders. DNA chips are known, for example, in US Patent 5,837,832.

The described invention further provides a composition of matter useful for prognosing the relapse of breast cancer patients. Said composition comprising at least one nucleic acid 18 base pairs in length of a segment of the nucleic acid sequence disclosed in SEQ ID NO: 2 to 5, and one or more substances taken from the group comprising :
1-5 mM Magnesium Chloride, 100-500 μM dNTP, 0.5-5 units of taq polymerase, bovine serum albumen, an oligomer in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto. It is preferred that said composition of matter comprises a buffer solution appropriate for the stabilisation of said nucleic acid in an

aqueous solution and enabling polymerase based reactions within said solution.. Suitable buffers are known in the art and commercially available.

The present invention further provides a method for conducting an assay in order to ascertain genetic and/or epigenetic parameters of the gene PITX2 and its promoter and regulatory elements. Most preferably the assay according to the following method is used in order to detect methylation within the gene PITX2 wherein said methylated nucleic acids are present in a solution further comprising an excess of background DNA, wherein the background DNA is present in between 100 to 1000 times the concentration of the DNA to be detected. Said method comprising contacting a nucleic acid sample obtained from said subject with at least one reagent or a series of reagents, wherein said reagent or series of reagents, distinguishes between methylated and non-methylated CpG dinucleotides within the target nucleic acid.

Preferably, said method comprises the following steps: In the first *step*, a sample of the tissue to be analysed is obtained. The source may be any suitable source, preferably, the source of the sample is selected from the group consisting of histological slides, biopsies, paraffin-embedded tissue, bodily fluids, plasma, serum, stool, urine, blood, nipple aspirate and combinations thereof. Preferably, the source is tumor tissue, biopsies, serum, urine, blood or nipple aspirate. The most preferred source, is the tumor sample, surgically removed from the patient or a biopsy sample of said patient.

The DNA is then isolated from the sample. Extraction may be by means that are standard to one skilled in the art, including the use of detergent lysates, sonification and vortexing with glass beads.. Once the nucleic acids have been extracted, the genomic double stranded DNA is used in the analysis.

In the second step of the method, the genomic DNA sample is treated in such a manner that cytosine bases which are unmethylated at the 5'-position are converted to uracil, thymine, or another base which is dissimilar to cytosine in terms of hybridisation behaviour. This will be understood as 'pretreatment' herein.

The above described treatment of genomic DNA is preferably carried out with bisulfite (hydrogen sulfite, disulfite) and subsequent alkaline hydrolysis which results in a conversion of non-methylated cytosine nucleobases to uracil or to another base which is dissimilar to

cytosine in terms of base pairing behaviour. Enclosing the DNA to be analysed in an agarose matrix, thereby preventing the diffusion and renaturation of the DNA (bisulfite only reacts with single-stranded DNA), and replacing all precipitation and purification steps with fast dialysis (Olek A, et al., A modified and improved method for bisulfite based cytosine methylation analysis, *Nucleic Acids Res.* 24:5064-6, 1996). It is further preferred that the bisulfite treatment is carried out in the presence of a radical scavenger or DNA denaturing agent.

In the third step of the method, fragments of the pretreated DNA are amplified. Wherein the source of the DNA is free DNA from serum, or DNA extracted from paraffin it is particularly preferred that the size of the amplificate fragment is between 100 and 200 base pairs in length, and wherein said DNA source is extracted from cellular sources (e.g. tissues, biopsies, cell lines) it is preferred that the amplificate is between 100 and 350 base pairs in length. It is particularly preferred that said amplificates comprise at least one 20 base pair sequence comprising at least three CpG dinucleotides. Said amplification is carried out using sets of primer oligonucleotides according to the present invention, and a preferably heat-stable polymerase. The amplification of several DNA segments can be carried out simultaneously in one and the same reaction vessel, in one embodiment of the method preferably six or more fragments are amplified simultaneously. Typically, the amplification is carried out using a polymerase chain reaction (PCR). The set of primer oligonucleotides includes at least two oligonucleotides whose sequences are each reverse complementary, identical, or hybridise under stringent or highly stringent conditions to an at least 18-base-pair long segment of the base sequences of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

In an alternate embodiment of the method, the methylation status of preselected CpG positions within the nucleic acid sequences comprising SEQ ID NO: 2 to SEQ ID NO: 5 may be detected by use of methylation-specific primer oligonucleotides. This technique (MSP) has been described in United States Patent No. 6,265,171 to Herman. The use of methylation status specific primers for the amplification of bisulfite treated DNA allows the differentiation between methylated and unmethylated nucleic acids. MSP primers pairs contain at least one primer which hybridises to a bisulfite treated CpG dinucleotide. Therefore, the sequence of said primers comprises at least one CpG , TpG or CpA dinucleotide. MSP primers specific for non-methylated DNA contain a "T" at the 3' position of the C position in the CpG. Preferably, therefore, the base sequence of said primers is required to comprise a sequence having a

length of at least 18 nucleotides which hybridises to a pretreated nucleic acid sequence according to SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto, wherein the base sequence of said oligomers comprises at least one CpG, tpG or Cpa dinucleotide. In this embodiment of the method according to the invention it is particularly preferred that the MSP primers comprise between 2 and 4 CpG , tpG or Cpa dinucleotides. It is further preferred that said dinucleotides are located within the 3' half of the primer e.g. wherein a primer is 18 bases in length the specified dinucleotides are located within the first 9 bases form the 3'end of the molecule. In addition to the CpG , tpG or Cpa dinucleotides it is further preferred that said primers should further comprise several bisulfite converted bases (i.e. cytosine converted to thymine, or on the hybridising strand, guanine converted to adenosine). In a further preferred embodiment said primers are designed so as to comprise no more than 2 cytosine or guanine bases.

In one embodiment of the method the primers may be selected form the group consisting to SEQ ID NO: 6 to SEQ ID NO: 10.

The fragments obtained by means of the amplification can carry a directly or indirectly detectable label. Preferred are labels in the form of fluorescence labels, radionuclides, or detachable molecule fragments having a typical mass which can be detected in a mass spectrometer. Where said labels are mass labels, it is preferred that the labelled amplificates have a single positive or negative net charge, allowing for better detectability in the mass spectrometer. The detection may be carried out and visualised by means of, *e.g.*, matrix assisted laser desorption/ionisation mass spectrometry (MALDI) or using electron spray mass spectrometry (ESI).

Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-TOF) is a very efficient development for the analysis of biomolecules (Karas & Hillenkamp, *Anal Chem.*, 60:2299-301, 1988). An analyte is embedded in a light-absorbing matrix. The matrix is evaporated by a short laser pulse thus transporting the analyte molecule into the vapour phase in an unfragmented manner. The analyte is ionised by collisions with matrix molecules. An applied voltage accelerates the ions into a field-free flight tube. Due to their different masses, the ions are accelerated at different rates. Smaller ions reach the detector sooner than bigger ones. MALDI-TOF spectrometry is well suited to the analysis of peptides and proteins. The analysis of nucleic acids is somewhat more difficult (Gut & Beck, *Current Innovations and*

Future Trends, 1:147-57, 1995). The sensitivity with respect to nucleic acid analysis is approximately 100-times less than for peptides, and decreases disproportionally with increasing fragment size. Moreover, for nucleic acids having a multiply negatively charged backbone, the ionisation process via the matrix is considerably less efficient. In MALDI-TOF spectrometry, the selection of the matrix plays an eminently important role. For the desorption of peptides, several very efficient matrixes have been found which produce a very fine crystallisation. There are now several responsive matrixes for DNA, however, the difference in sensitivity between peptides and nucleic acids has not been reduced. This difference in sensitivity can be reduced, however, by chemically modifying the DNA in such a manner that it becomes more similar to a peptide. For example, phosphorothioate nucleic acids, in which the usual phosphates of the backbone are substituted with thiophosphates, can be converted into a charge-neutral DNA using simple alkylation chemistry (Gut & Beck, *Nucleic Acids Res.* 23: 1367-73, 1995). The coupling of a charge tag to this modified DNA results in an increase in MALDI-TOF sensitivity to the same level as that found for peptides. A further advantage of charge tagging is the increased stability of the analysis against impurities, which makes the detection of unmodified substrates considerably more difficult.

In a particularly preferred embodiment of the method the amplification of step three is carried out in the presence of at least one species of blocker oligonucleotides. The use of such blocker oligonucleotides has been described by Yu et al., *BioTechniques* 23:714-720, 1997. The use of blocking oligonucleotides enables the improved specificity of the amplification of a subpopulation of nucleic acids. Blocking probes hybridised to a nucleic acid suppress, or hinder the polymerase mediated amplification of said nucleic acid. In one embodiment of the method blocking oligonucleotides are designed so as to hybridise to background DNA. In a further embodiment of the method said oligonucleotides are designed so as to hinder or suppress the amplification of unmethylated nucleic acids as opposed to methylated nucleic acids or vice versa.

Blocking probe oligonucleotides are hybridised to the bisulfite treated nucleic acid concurrently with the PCR primers. PCR amplification of the nucleic acid is terminated at the 5' position of the blocking probe, such that amplification of a nucleic acid is suppressed where the complementary sequence to the blocking probe is present. The probes may be designed to hybridise to the bisulfite treated nucleic acid in a methylation status specific manner. For example, for detection of methylated nucleic acids within a population of unmethylated

nucleic acids, suppression of the amplification of nucleic acids which are unmethylated at the position in question would be carried out by the use of blocking probes comprising a 'TpG' at the position in question, as opposed to a 'CpG.' In one embodiment of the method the sequence of said blocking oligonucleotides should be identical or complementary to molecule is complementary or identical to a sequence at least 18 base pairs in length selected from the group consisting of SEQ ID NOS: 2 to 5, preferably comprising one or more CpG, TpG or CpA dinucleotides . In one embodiment of the method the sequence of said oligonucleotides is selected from the group consisting SEQ ID NO: 15 and SEQ ID NO: 16 and sequences complementary thereto.

For PCR methods using blocker oligonucleotides, efficient disruption of polymerase-mediated amplification requires that blocker oligonucleotides not be elongated by the polymerase. Preferably, this is achieved through the use of blockers that are 3'-deoxyoligonucleotides, or oligonucleotides derivitised at the 3' position with other than a "free" hydroxyl group. For example, 3'-O-acetyl oligonucleotides are representative of a preferred class of blocker molecule.

Additionally, polymerase-mediated decomposition of the blocker oligonucleotides should be precluded. Preferably, such preclusion comprises either use of a polymerase lacking 5'-3' exonuclease activity, or use of modified blocker oligonucleotides having, for example, thioate bridges at the 5'-termini thereof that render the blocker molecule nuclease-resistant. Particular applications may not require such 5' modifications of the blocker. For example, if the blocker- and primer-binding sites overlap, thereby precluding binding of the primer (*e.g.*, with excess blocker), degradation of the blocker oligonucleotide will be substantially precluded. This is because the polymerase will not extend the primer toward, and through (in the 5'-3' direction) the blocker - a process that normally results in degradation of the hybridised blocker oligonucleotide.

A particularly preferred blocker/PCR embodiment, for purposes of the present invention and as implemented herein, comprises the use of peptide nucleic acid (PNA) oligomers as blocking oligonucleotides. Such PNA blocker oligomers are ideally suited, because they are neither decomposed nor extended by the polymerase.

In one embodiment of the method, the binding site of the blocking oligonucleotide is identical to, or overlaps with that of the primer and thereby hinders the hybridisation of the primer to its binding site. In a further preferred embodiment of the method, two or more such blocking oligonucleotides are used. In a particularly preferred embodiment, the hybridisation of one of the blocking oligonucleotides hinders the hybridisation of a forward primer, and the hybridisation of another of the probe (blocker) oligonucleotides hinders the hybridisation of a reverse primer that binds to the amplificate product of said forward primer.

In an alternative embodiment of the method, the blocking oligonucleotide hybridises to a location between the reverse and forward primer positions of the treated background DNA, thereby hindering the elongation of the primer oligonucleotides.

It is particularly preferred that the blocking oligonucleotides are present in at least 5 times the concentration of the primers.

In the fourth step of the method, the amplificates obtained during the third step of the method are analysed in order to ascertain the methylation status of the CpG dinucleotides prior to the treatment.

In embodiments where the amplificates were obtained by means of MSP amplification and/or blocking oligonucleotides, the presence or absence of an amplificate is in itself indicative of the methylation state of the CpG positions covered by the primers and or blocking oligonucleotide, according to the base sequences thereof.. All possible known molecular biological methods may be used for this detection, including, but not limited to gel electrophoresis, sequencing, liquid chromatography, hybridisations, real time PCR analysis or combinations thereof. This step of the method further acts as a qualitative control of the preceding steps.

In the fourth step of the method amplificates obtained by means of both standard and methylation specific PCR are further analysed in order to determine the CpG methylation status of the genomic DNA isolated in the first step of the method. This may be carried out by means of hybridization-based methods such as, but not limited to, array technology and probe based technologies as well as by means of techniques such as sequencing and template directed extension.

In one embodiment of the method, the amplificates synthesised in step three are subsequently hybridised to an array or a set of oligonucleotides and/or PNA probes. In this context, the hybridisation takes place in the following manner: the set of probes used during the hybridisation is preferably composed of at least 2 oligonucleotides or PNA-oligomers; in the process, the amplificates serve as probes which hybridise to oligonucleotides previously bonded to a solid phase; the non-hybridised fragments are subsequently removed; said oligonucleotides contain at least one base sequence having a length of at least 9 nucleotides which is reverse complementary or identical to a segment of the base sequences specified in the SEQ ID NO: 2 to SEQ ID NO: 5; and the segment comprises at least one CpG , TpG or CpA dinucleotide.

In a preferred embodiment, said dinucleotide is present in the central third of the oligomer. For example, wherein the oligomer comprises one CpG dinucleotide, said dinucleotide is preferably the fifth to ninth nucleotide from the 5'-end of a 13-mer. One oligonucleotide exists for the analysis of each CpG dinucleotide within the sequence according to SEQ ID NO: 1, and the equivalent positions within SEQ ID NOS: 2 to 5. Said oligonucleotides may also be present in the form of peptide nucleic acids. The non-hybridised amplificates are then removed. The hybridised amplificates are detected. In this context, it is preferred that labels attached to the amplificates are identifiable at each position of the solid phase at which an oligonucleotide sequence is located.

In yet a further embodiment of the method, the genomic methylation status of the CpG positions may be ascertained by means of oligonucleotide probes that are hybridised to the bisulfite treated DNA concurrently with the PCR amplification primers (wherein said primers may either be methylation specific or standard).

A particularly preferred embodiment of this method is the use of fluorescence-based Real Time Quantitative PCR (Heid et al., *Genome Res.* 6:986-994, 1996; *also see* United States Patent No. 6,331,393). There are two preferred embodiments of utilising this method. One embodiment, known as the TaqMan™ assay employs a dual-labelled fluorescent oligonucleotide probe. The TaqMan™ PCR reaction employs the use of a non-extendible interrogating oligonucleotide, called a TaqMan™ probe, which is designed to hybridise to a CpG-rich sequence located between the forward and reverse amplification primers. The

TaqMan™ probe further comprises a fluorescent "reporter moiety" and a "quencher moiety" covalently bound to linker moieties (*e.g.*, phosphoramidites) attached to the nucleotides of the TaqMan™ oligonucleotide. Hybridised probes are displaced and broken down by the polymerase of the amplification reaction thereby leading to an increase in fluorescence. For analysis of methylation within nucleic acids subsequent to bisulfite treatment, it is required that the probe be methylation specific, as described in United States Patent No. 6,331,393, (hereby incorporated by reference in its entirety) also known as the MethylLight assay. The second preferred embodiment of this technology is the use of dual-probe technology (Lightcycler®), each carrying donor or recipient fluorescent moieties, hybridisation of two probes in proximity to each other is indicated by an increase or fluorescent amplification primers. Both these techniques may be adapted in a manner suitable for use with bisulfite treated DNA, and moreover for methylation analysis within CpG dinucleotides.
Also any combination of these probes or combinations of these probes with other known probes may be used.

In a further preferred embodiment of the method, the fourth step of the method comprises the use of template-directed oligonucleotide extension, such as MS-SNuPE as described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997. In said embodiment it is preferred that the methylation specific single nucleotide extension primer (MS-SNuPE preimer) is identical or complementary to a sequence at least nine but preferably no more than twenty five nucleotides in length of one or more of the sequences taken from the group of SEQ ID NO: 2 to SEQ ID NO: 5. However it is preferred to use fluorescently labelled nucleotides, instead of radiolabelled nucleotides.

In yet a further embodiment of the method, the fourth step of the method comprises sequencing and subsequent sequence analysis of the amplificate generated in the third step of the method (Sanger F., et al., *Proc Natl Acad Sci USA* 74:5463-5467, 1977).

Additional embodiments of the invention provide a method for the analysis of the methylation status of genomic DNA according to the invention (SEQ ID NO: 1) without the need for pretreatment.

In the *first step* of such additional embodiments, the genomic DNA sample is isolated from tissue or cellular sources. Preferably, such sources include cell lines, histological slides, body

fluids, or tissue embedded in paraffin. Extraction may be by means that are standard to one skilled in the art, including but not limited to the use of detergent lysates, sonification and vortexing with glass beads. Once the nucleic acids have been extracted, the genomic double-stranded DNA is used in the analysis.

In a preferred embodiment, the DNA may be cleaved prior to the treatment, and this may be by any means standard in the state of the art, in particular with methylation-sensitive restriction endonucleases.

In the *second step*, the DNA is then digested with one or more methylation sensitive restriction enzymes. The digestion is carried out such that hydrolysis of the DNA at the restriction site is informative of the methylation status of a specific CpG dinucleotide.

In the *third step*, which is optional but a preferred embodiment, the restriction fragments are amplified. This is preferably carried out using a polymerase chain reaction, and said amplificates may carry suitable detectable labels as discussed above, namely fluorophore labels, radionuclides and mass labels.

In the *final step* the amplificates are detected. The detection may be by any means standard in the art, for example, but not limited to, gel electrophoresis analysis, hybridisation analysis, incorporation of detectable tags within the PCR products, DNA array analysis, MALDI or ESI analysis.

The present invention enables prognosis of events which are disadvantageous to patients or individuals in which important genetic and/or epigenetic parameters within the PITX2 gene and its promoter or regulatory elements may be used as prognostic markers for breast cancer relapse or as 'adjuvant marker' for prediction of need of additional treatment besides of endocrine monotherapy. Said parameters obtained by means of the present invention may be compared to another set of genetic and/or epigenetic parameters, the differences serving as the basis for a prognosis of events which are disadvantageous to patients or individuals.

Specifically, the present invention provides for prognostic cancer relapse assays based on measurement of differential methylation of PITX2 CpG dinucleotide sequences. Preferred

gene sequences useful to measure such differential methylation are represented herein by SEQ ID NOS: 1 to 5. Typically, such assays involve obtaining a tissue sample from a test tissue, performing an assay to measure the methylation status of at least one of the inventive PITX2-specific CpG dinucleotide sequences derived from the tissue sample, relative to a control sample, and making a diagnosis or prognosis or prediction based thereon.

In particular preferred embodiments, inventive oligomers are used to assess PITX2 specific CpG dinucleotide methylation status, such as those based on SEQ ID NOS: 1 to 5, or arrays thereof, as well as a kit based thereon are useful for the prognosis of breast cancer relapse and/or the survival of a patient diagnosed with breast cancer, preferably under endocrine treatment since surgical removal of the tumor.

Moreover, an additional aspect of the present invention is a kit comprising, for example: a bisulfite-containing reagent as well as at least one oligonucleotide whose sequences in each case correspond, are complementary, or hybridise under stringent or highly stringent conditions to a 18-base long segment of the sequences SEQ ID NOS: 1 to 5. Said kit may further comprise instructions for carrying out and evaluating the described method. In a further preferred embodiment, said kit may further comprise standard reagents for performing a CpG position-specific methylation analysis, wherein said analysis comprises one or more of the following techniques: MS-SNuPE, MSP, MethyLight™, HeavyMethyl™, COBRA, and nucleic acid sequencing. However, a kit along the lines of the present invention can also contain only part of the aforementioned components.

Typical reagents (*e.g.*, as might be found in a typical COBRA-based kit) for COBRA analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); restriction enzyme and appropriate buffer; gene-hybridisation oligo; control hybridisation oligo; kinase labelling kit for oligo probe; and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery reagents or kits (*e.g.*, precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (*e.g.*, as might be found in a typical MethyLight®-based kit) for MethyLight® analysis may include, but are not limited to: PCR primers for specific gene (or

methylation-altered DNA sequence or CpG island); TaqMan® probes; optimised PCR buffers and deoxynucleotides; and Taq polymerase.

Typical reagents (*e.g.*, as might be found in a typical Ms-SNuPE-based kit) for Ms-SNuPE analysis may include, but are not limited to: PCR primers for specific gene (or methylation-altered DNA sequence or CpG island); optimised PCR buffers and deoxynucleotides; gel extraction kit; positive control primers; Ms-SNuPE primers for specific gene; reaction buffer (for the Ms-SNuPE reaction); and radioactive nucleotides. Additionally, bisulfite conversion reagents may include: DNA denaturation buffer; sulfonation buffer; DNA recovery regents or kit (*e.g.*, precipitation, ultrafiltration, affinity column); desulfonation buffer; and DNA recovery components.

Typical reagents (*e.g.*, as might be found in a typical MSP-based kit) for MSP analysis may include, but are not limited to: methylated and unmethylated PCR primers for specific gene (or methylation-altered DNA sequence or CpG island), optimised PCR buffers and deoxynucleotides, and specific probes.

Definitions:

In the context of the present invention, the term "CpG island" refers to a contiguous region of genomic DNA that satisfies the criteria of (1) having a frequency of CpG dinucleotides corresponding to an "Observed/Expected Ratio" >0.6, and (2) having a "GC Content" >0.5. CpG islands are typically, but not always, between about 0.2 to about 1 kb in length.

In the context of the present invention, the term "methylation" refers to the presence or absence of 5-methylcytosine ("5-mCyt") at one or a plurality of CpG dinucleotides within a DNA sequence.

In the context of the present invention the term "methylation state" is taken to mean the degree of methylation present in a nucleic acid of interest, this may be expressed in absolute or relative terms i.e. as a percentage or other numerical value or by comparison to another tissue and therein described as hypermethylated, hypomethylated or as having significantly similar or identical methylation status.

In the context of the present invention, the term "hemi-methylation" or "hemimethylation" refers to the methylation state of a palindromic CpG methylation site, where only a single cytosine in one of the two CpG dinucleotide sequences of the double stranded CpG methylation site is methylated (*e.g.*, 5'-NNC^{M}GNN-3' (top strand): 3'-NNGCNN-5' (bottom strand)).

In the context of the present invention, the term "hypermethylation" refers to the average methylation state corresponding to an *increased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

In the context of the present invention, the term "hypomethylation" refers to the average methylation state corresponding to a *decreased* presence of 5-mCyt at one or a plurality of CpG dinucleotides within a DNA sequence of a test DNA sample, relative to the amount of 5-mCyt found at corresponding CpG dinucleotides within a normal control DNA sample.

In the context of the present invention, the term "microarray" refers broadly to both "DNA microarrays," and 'DNA chip(s),' as recognised in the art, encompasses all art-recognised solid supports, and encompasses all methods for affixing nucleic acid molecules thereto or synthesis of nucleic acids thereon.

"Genetic parameters" are mutations and polymorphisms of genes and sequences further required for their regulation. To be designated as mutations are, in particular, insertions, deletions, point mutations, inversions and polymorphisms and, particularly preferred, SNPs (single nucleotide polymorphisms).

"Epigenetic modifications" or "epigenetic parameters" are modifications of DNA bases of genomic DNA and sequences further required for their regulation, in particular, cytosine methylations thereof. Further epigenetic parameters include, for example, the acetylation of histones which, however, cannot be directly analysed using the described method but which, in turn, correlate with the DNA methylation.

In the context of the present invention, the term "bisulfite reagent" refers to a reagent comprising bisulfite, disulfite, hydrogen sulfite or combinations thereof, useful as disclosed herein to distinguish between methylated and unmethylated CpG dinucleotide sequences.

In the context of the present invention, the term "Methylation assay" refers to any assay for determining the methylation state of one or more CpG dinucleotide sequences within a sequence of DNA.

In the context of the present invention, the term "MS.AP-PCR" (Methylation-Sensitive Arbitrarily-Primed Polymerase Chain Reaction) refers to the art-recognised technology that allows for a global scan of the genome using CG-rich primers to focus on the regions most likely to contain CpG dinucleotides, and described by Gonzalgo et al., *Cancer Research* 57:594-599, 1997.

In the context of the present invention, the term "MethyLight" refers to the art-recognised fluorescence-based real-time PCR technique described by Eads et al., *Cancer Res.* 59:2302-2306, 1999.

In the context of the present invention, the term "HeavyMethyl™" assay, in the embodiment thereof implemented herein, refers to a HeavyMethyl™ MethylLight assay, which is a variation of the MethylLight assay, wherein the MethylLight assay is combined with methylation specific *blocking* probes covering CpG positions between the amplification primers.

The term "Ms-SNuPE" (Methylation-sensitive Single Nucleotide Primer Extension) refers to the art-recognized assay described by Gonzalgo & Jones, *Nucleic Acids Res.* 25:2529-2531, 1997.

In the context of the present invention the term "MSP" (Methylation-specific PCR) refers to the art-recognised methylation assay described by Herman et al. *Proc. Natl. Acad. Sci. USA* 93:9821-9826, 1996, and by US Patent No. 5,786,146.

In the context of the present invention the term "COBRA" (Combined Bisulfite Restriction Analysis) refers to the art-recognized methylation assay described by Xiong & Laird, *Nucleic Acids Res.* 25:2532-2534, 1997.

In the context of the present invention the term "hybridisation" is to be understood as a bond of an oligonucleotide to a complementary sequence along the lines of the Watson-Crick base pairings in the sample DNA, forming a duplex structure.

"Stringent hybridisation conditions," as defined herein, involve hybridising at 68°C in 5x SSC/5x Denhardt's solution/1.0% SDS, and washing in 0.2x SSC/0.1% SDS at room temperature, or involve the art-recognised equivalent thereof (e.g., conditions in which a hybridisation is carried out at 60°C in 2.5 x SSC buffer, followed by several washing steps at 37°C in a low buffer concentration, and remains stable). Moderately stringent conditions, as defined herein, involve including washing in 3x SSC at 42°C, or the art-recognised equivalent thereof. The parameters of salt concentration and temperature can be varied to achieve the optimal level of identity between the probe and the target nucleic acid. Guidance regarding

such conditions is available in the art, for example, by Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, N.Y.; and Ausubel et al. (eds.), 1995, Current Protocols in Molecular Biology, (John Wiley & Sons, N.Y.) at Unit 2.10.

"Background DNA" as used herein refers to any nucleic acids which originate from sources other than colon cells.

In the context of this application "survival" is meant to describe the time from diagnosis or start of treatment to an endpoint, which may be either the time of death (considering any reason for death or only death from breast cancer), or the time of recurrence of breast cancer (for example in form of metastases), which may be local or distant, or the time of occurrence of any breast cancer associated disease. Therefore "predicting the survival" is meant to comprise predicting the disease free survival, as well as the overall survival or any other consideration of time between diagnosis and endpoint of treatment.

Throughout this invention it is preferred that said survival is characterized as the disease free or the overall survival. It is especially preferred that survival is understood as disease free survival. Disease free survival is understood as absence of recurrence of cancer (local or distant) .

The terms "endocrine therapy" or "endocrine treatment" is meant to comprise any therapy, treatment or treatments targeting the estrogen receptor pathway or estrogen synthesis pathway or estrogen conversion pathway, which is involved in estrogen metabolism, production or secretion. Said treatments include, but are not limited to estrogen receptor modulators, estrogen receptor down-regulators, aromatase inhibitors, ovarian ablation, LHRH analogues and other centrally acting drugs influencing estrogen production.

The term "monotherapy" is used to explain that no other treatment is given in addition or to support said monotherapy.

In the context of the present invention the term "regulatory region" of a gene is taken to mean nucleotide sequences which affect the expression of a gene. Said regulatory regions may be located within, proximal or distal to said gene. Said regulatory regions include but are not limited to constitutive promoters, tissue-specific promoters, developmental-specific

promoters, inducible promoters and the like. Promoter regulatory elements may also include certain enhancer sequence elements that control transcriptional or translational efficiency of the gene.

In the context of the present invention the term "chemotherapy" is taken to mean the use of drugs or chemical substances to treat cancer. This definition excludes radiation therapy (treatment with high energy rays or particles), hormone therapy (treatment with hormones or hormone analogues (synthetic substitutes) and surgical treatment.

In the context of the present invention the term "adjuvant treatment" is taken to mean a therapy of a cancer patient immediately following an initial non chemotherapeutical therapy, e.g. surgery. In general, the purpose of an adjuvant therapy is to provide a significantly smaller risk of recurrences compared without the adjuvant therapy.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples and figures serve only to illustrate the invention and is not intended to limit the invention within the principles and scope of the broadest interpretations and equivalent configurations thereof.

In the sequence protocol and the Figures,

SEQ ID NO: 1 shows the sequence of the human gene PITX2,
SEQ ID NOS: 2 to 5 show chemically pretreated sequences of the gene PITX2,
SEQ ID NOS: 6 to 9 show the sequences of primers and probes according to PITX2 used in the example,
SEQ ID NOS: 10 to 12 show the sequences of primers and probes according to a control gene used in the example.

FIGURES

Figure 1 shows a preferred application of the method according to the invention. The X axis shows the tumour(s) mass, wherein the line '3' shows the limit of detectability. The Y-axis shows time. Accordingly said figure illustrates a simplified model of endocrine treatment of

an Stage 1-3 breast tumour wherein primary treatment was surgery (at point 1), followed by adjuvant therapy with Tamoxifen, as an example for an endocrine treatment. In a first scenario a patient without relapse during endocrine treatment (4) is shown as remaining below the limit of detectability for the duration of the observation. A patient with relapse of the cancer (5) has a period of disease free survival (2) followed by relapse when the carcinoma mass reaches the level of detectability.

Figure 2 shows the Kaplan-Meier estimated disease-free survival curves for a CpG position of the PITX2 gene by means of Real-Time methylation specific probe analysis. The lower plot shows the proportion of disease free patients in the population with above median methylation levels, the upper plot shows shows the proportion of disease free patients in the population with below median methylation levels. The X axis shows the disease free survival times of the patients in months, and the Y- axis shows the proportion of disease free survival patients.

EXAMPLE

Real time Quantitative methylation analysis

Genomic DNA was analyzed using the Real Time PCR technique after bisulfite conversion. In this analysis four oligonucleotides were used in each reaction. Two non methylation specific PCR primers were used to amplify a segment of the treated genomic DNA containing a methylation variable oligonucleotide probe binding site. Two oligonucleotide probes competitively hybridise to the binding site, one specific for the methylated verison of the binding site, the other specific to the unmethlyated version of the binding site. Accordingly, one of the probes comprises a CpG at the methylation variable position (i.e. anneals to methylated bisulphite treated sites) and the other comprises a TpG at said positon (i.e. anneals to unmethylated bisulphite treated sites). Each species of probe is labelled with a 5' fluorescent reporter dye and a 3' quencher dye wherein the CpG and TpG oligonucleotides are labelled with different dyes.

The reactions are calibrated by reference to DNA standards of known methylation levels in order to quantify the levels of methlyation within the sample. The DNA standards were composed of bisulfite treated phi29 amplified genomic DNA (i.e. unmethlyated), and/or phi29 amplified genomic DNA treated with Sss1 methylase enzyme (thereby methylating each CpG position in the sample), which is then treated with bisulfite solution. Seven

different reference standards were used with 0%, (i.e. phi29 amplified genomic DNA only), 5%, 10%, 25%, 50%, 75% and 100% (i.e. phi29 Sss1 treated genomic only).

The amount of sample DNA amplified is quantified by reference to the gene (ß-actin (ACTB)) to normalize for input DNA. For standardization the primers and the probe for analysis of the ACTB gene lack CpG dinucleotides so that amplification is possible regardless of methylation levels. As there are no methylation variable positions, only one probe oligonucleotide is required.

The following oligonucleotides were used in the reaction:

Primer: TGGTGATGGAGGAGGTTTAGTAAGT (SEQ ID NO: 10)

Primer: AACCAATAAAACCTACTCCTCCCTTAA (SEQ ID NO: 11)

Probe: 6FAM-ACCACCACCCAACACACAATAACAAACACA-TAMRA or Dabcyl (SEQ ID NO: 12)

The extent of methylation at a specific locus was determined by the following formula:

methylation rate= 100 * I (CG) / (I(CG) + I(TG))

(I = Intensity of the fluorescence of CG-probe or TG-probe)

Gene PITX2 (SEQ ID 1, und 2-5)

Primers:
PITX2R02: GTAGGGGAGGGAAGTAGATGTT (SEQ ID NO: 6)
PITX2Q02: TTCTAATCCTCCTTTCCACAATAA (SEQ ID NO: 7)
Amplificate length : 143 bp
Probes:
PITX2cg1: FAM-AGTCGGAGTCGGGAGAGCGA-Darquencher (SEQ ID NO: 8)
PITX2tg1: YAKIMA YELLOW-AGTTGGAGTTGGGAGAGTGAAAGGAGA-Darquencher (SEQ ID NO: 9)

PCR components: 3 mM MgCl2 buffer, 10x buffer, Hotstart TAQ

Program (45 cycles): 95 °C, 10 min
95 °C, 15 sec
62 °C, 1 min

Figure 2 shows the Kaplan-Meier estimated disease-free survival curves for a CpG position of the PITX2 gene by means of Real-Time methylation specific probe analysis. The lower plot shows the proportion of disease free patients in the population with above median methylation levels, the upper plot shows shows the proportion of disease free patients in the population with below median methylation levels. The X axis shows the disease free survival times of the patients in months, and the Y- axis shows the proportion of disease free survival patients. The p-value (probability that the observed distribution occurred by chance) was calculated as 0.0031, thereby confirming the data obtained by means of array analysis.

CLAIMS:

1. A method for characterising a cell proliferative disorder of the breast tissues and/or **predicting the survival** of a patient diagnosed with said disorder, comprising the steps of:
(a) obtaining one or more biological samples from the patient; and
(b) detecting the level of expression of a polypeptide expressed from the PITX2 gene

2. The method according to claim 1 further comprising
(c) determining therefrom the survival of said patient, characteristics of said cell proliferative disorder, and/or prognosis of said patient

3. The method according to claim 1 further comprising
(d) determining a suitable treatment regimen for the subject

4. The method of claim 1, wherein said patient is characterized by being subject to adjuvant endocrine therapy comprising one or more treatments which target the estrogen receptor pathway or are involved in estrogen metabolism, production or secretion.

5. The method of claim 1, wherein said breast cell proliferative disorders are taken from the group comprising ductal carcinoma *in situ*, invasive ductal carcinoma, invasive lobular carcinoma, lobular carcinoma *in situ,* comedocarcinoma, inflammatory carcinoma, mucinous carcinoma, scirrhous carcinoma, colloid carcinoma, tubular carcinoma, medullary carcinoma, metaplastic carcinoma, and papillary carcinoma and papillary carcinoma *in situ*, undifferentiated or anaplastic carcinoma and Paget's disease of the breast.

6. The method of claim 1, wherein said detection is afforded by performing an immunoassay, in particular by an ELISA.

7. The method of claim 6, wherein said immunoassay is a radioimmunoassay.

8. A method of predicting survival of a patient diagnosed with a cell proliferative disorder of the breast, comprising the steps of:

a) obtaining one or more biological test samples from said patient; and

b) contacting said sample with an antibody immunoreactive with the PITX2 polypeptide to form an immunocomplex;

c) detecting said immunocomplex;

d) comparing the quantity of said immunocomplex to the quantity of immunocomplex formed under identical conditions with the same antibody and a control sample from one or more patients with a known prognosis; wherein a decrease in quantity of said immunocomplex in the sample from said subject relative to said control sample is indicative of a bad prognosis.

9. The method of claim 8, wherein said immunocomplex is detected in a Western blot assay.

10. The method of claim 8, wherein said immunocomplex is detected in an ELISA.

11. The method of claim 1, wherein said detection is afforded by expression analysis.

12. The method of claim 11, comprising detecting the presence or absence of mRNA encoding a PITX2 polypeptide in a sample from a patient, wherein a decreased concentration of said mRNA below the concentration determined for an individual known to have a good prognosis indicates a bad prognosis.

13. The method of claim 11, comprising the steps of:

a) providing a polynucleotide probe which specifically hybridises or is identical to a polynucleotide consisting of SEQ ID NO: 1,

(b) incubating said sample with said polynucleotide probe under high stringency conditions to form a specific hybridisation complex between an mRNA and said probe;

(c) detecting said hybridisation complex.

14. The method according to claim 13 wherein the detecting step further comprises the steps of:

a) producing a cDNA from mRNA in the sample;

b) providing two oligonucleotides which specifically hybridise to regions flanking a segment of the cDNA;

c) performing a polymerase chain reaction on the cDNA of step a) using the

oligonucleotides of step b) as primers to amplify the cDNA segment; and
d) detecting the amplified cDNA segment.

15. Use of a polypeptide expressed from the PITX2 gene for differentiating or distinguishing between patients diagnosed with breast cancer, which have a good survival prognosis and patients which have a bad survival prognosis.

16. Use of a polypeptide expressed from the PITX2 gene for prediction of survival of a patient diagnosed with a cell proliferative disorder of the breast.

17. The method of claim 1 wherein said detection comprises determining the genetic parameters of the gene PITX2, its promoter and/or regulatory elements.

18. The method of claim 1 wherein said detection comprises determining the epigenetic parameters of the gene PITX2, its promoter and/or regulatory elements.

19. The method of claim 1, wherein said detection comprises determining the **methylation** status of one or more CpG positions of a target nucleic acid within the gene PITX2, its promoter and/or regulatory elements, in particular through the methylation analysis of a genomic DNA sequence according to SEQ ID NO: 1.

20. The method of claim 19, wherein the methylation analysis is afforded by contacting said target nucleic acid with one or more agents that convert cytosine bases that are unmethylated at the 5'-position thereof to a base that is detectably dissimilar to cytosine in terms of hybridization properties.

21. The method of claim 20, wherein contacting said target nucleic acids with one or more agents comprises use of a solution selected from the group consisting of bisulfite, hydrogen sulfite, disulfite, and combinations thereof.

22. A nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated genomic DNA according to one of the sequences taken from the group comprising SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

23. An oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

24. The oligomer as recited in Claim 23; wherein the base sequence includes at least one CpG, **t**pG or Cp**a** dinucleotide.

25. The oligomer as recited in Claim 24; characterised in that the cytosine of the CpG dinucleotide is located approximately in the middle third of the oligomer.

26. A set of oligomers, comprising at least two oligomers according to any of claims 23 to 25.

27. A set of at least two oligonucleotides as recited in Claims 23 to 26, which can be used as primer oligonucleotides for the amplification of DNA sequences of one of SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

28. Use of a set of oligomer probes comprising at least ten of the oligomers according to any of claims 24 to 27 for detecting the cytosine methylation state and/or single nucleotide polymorphisms (SNPs) within one of the sequences according to SEQ ID NO: 1, and sequences complementary thereto.

29. A method for manufacturing an arrangement of different oligomers (array) fixed to a carrier material for analysing diseases associated with the methylation state of the CpG dinucleotides of one of SEQ ID NO: 1, and sequences complementary thereto wherein at least one oligomer according to any of the claims 23 through 27 is coupled to a solid phase.

30. A composition of matter comprising the following:

- a nucleic acid comprising a sequence at least 18 bases in length of a segment of the chemically pretreated genomic DNA according to one of the sequences taken from the group comprising SEQ ID NO: 1 to SEQ ID NO: 5 and sequences complementary

thereto, and

- a buffer comprising at least one of the following substances: 1 to 5 mM Magnesium Chloride, 100-500 μM dNTP, 0.5-5 units of taq polymerase, an oligomer, in particular an oligonucleotide or peptide nucleic acid (PNA)-oligomer, said oligomer comprising in each case at least one base sequence having a length of at least 9 nucleotides which is complementary to, or hybridises under moderately stringent or stringent conditions to a pretreated genomic DNA according to one of the SEQ ID NO: 2 to SEQ ID NO: 5 and sequences complementary thereto.

35. Use of the gene PITX2, its promoter and/or regulatory elements for detecting the survival od patients diagnosed with a cell proliferative disease.

36. A method for detecting the survival of patients diagnosed with a cell proliferative disease **according to claim 19**, comprising:

a) obtaining, from a subject, a biological sample having subject genomic DNA;

b) treating the genomic DNA, or a fragment thereof, with one or more reagents to convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridisation properties;

c) contacting the treated genomic DNA, or the treated fragment thereof, with an amplification enzyme and at least two primers comprising, in each case a contiguous sequence at least 18 nucleotides in length that is complementary to, or hybridises under moderately stringent or stringent conditions to a sequence selected from the group consisting of SEQ ID NOS: 2 to 5, and complements thereof, wherein the treated DNA or a fragment thereof is either amplified to produce one or more amplificates, or is not amplified; and

d) determining, based on the presence or absence of, or on a property of said amplificate, the methylation state of at least one CpG dinucleotide sequence of SEQ ID NO: 1, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences of SEQ ID NO: 1.

37. A method for detecting the survival of patients diagnosed with a cell proliferative disease **according to claim 19**, comprising the following steps of

a) obtaining, from a subject, a biological sample having subject genomic DNA;

b) treating the genomic DNA, or a fragment thereof, with one or more reagents to

convert 5-position unmethylated cytosine bases to uracil or to another base that is detectably dissimilar to cytosine in terms of hybridisation properties;

c) amplifying one or more fragments of the treated DNA such that only DNA originating from colon or colon cell proliferative disorder cells are amplified

d) detecting the amplificates or characteristics thereof and thereby deducing on the presence or absence of a colon cell proliferative disorder.

38. The method of one of claims 36 or 37, wherein in step a) the biological sample obtained from the subject is selected from the group consisting of histological slides, biopsies, paraffin-embedded tissue, bodily fluids, serum, plasma, stool, urine, blood, nipple aspirate and combinations thereof.

39. A method for detecting a colon cell proliferative disorder according to claim 17, comprising:

a) obtaining, from a subject, a biological sample having subject genomic DNA;

b) extracting the genomic DNA;

c) contacting the genomic DNA, or a fragment thereof, comprising SEQ ID NO:1 or a sequence that hybridises under stringent conditions to SEQ ID NO:1, with one or more methylation-sensitive restriction enzymes, wherein the genomic DNA is either digested thereby to produce digestion fragments, or is not digested thereby; and

d) determining, based on a presence or absence of, or on property of at least one such fragment, the methylation state of at least one CpG dinucleotide sequence of SEQ ID NO: 1, or an average, or a value reflecting an average methylation state of a plurality of CpG dinucleotide sequences of SEQ ID NO: 1, whereby at least one of detecting the prostate cell proliferative disorder, or distinguishing between a transitional and a peripheral zone of origin of the prostate cell proliferative disorder is, at least in part, afforded.

Figure 1

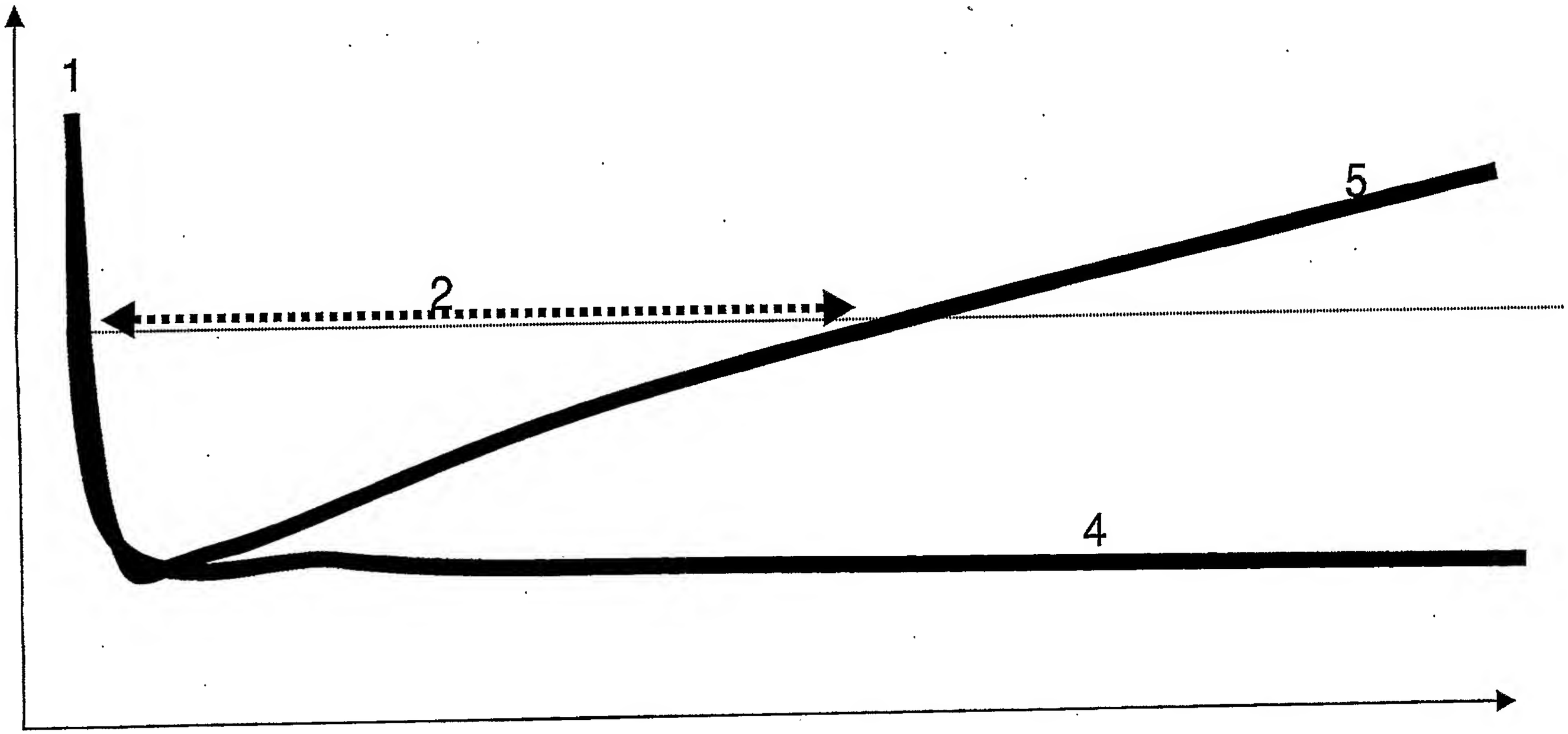
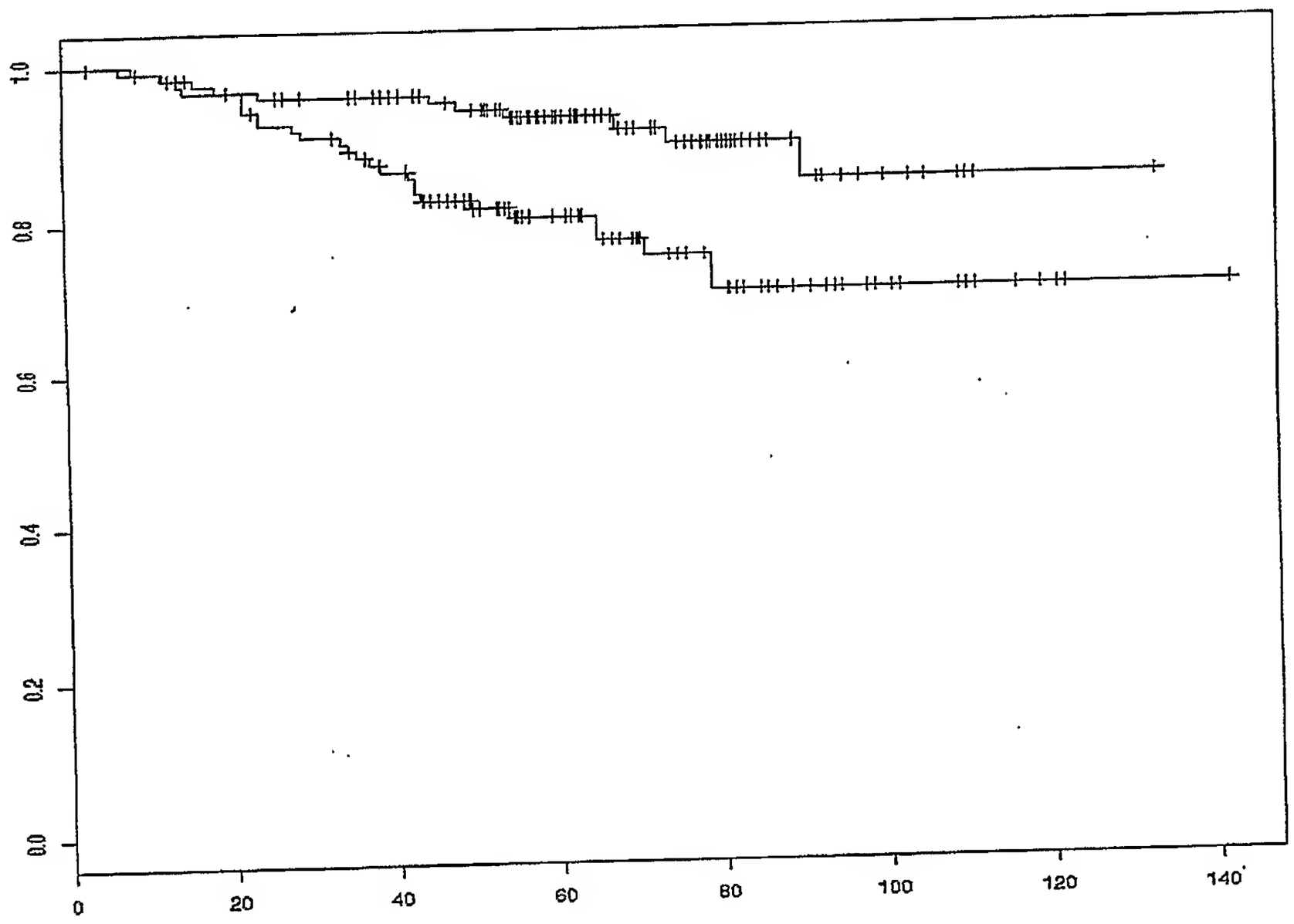


Figure 2



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